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Gene encoding polygalacturonase inhibitor in apple fruit is developmentally regulated and activated by wounding and fungal infection

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Abstract

A cDNA encoding polygalacturonase-inhibiting protein (PGIP) from mature apple fruit has been cloned and characterized. The open reading frame encodes a polypeptide of 330 amino acids, in which 24 amino acids at the N-terminus comprise the signal peptide. Apple PGIP contains 10 imperfect leucine-rich repeat sequence motifs averaging 24 amino acids in length. In addition to the 1.3 kb PGIP transcript, the cloned cDNA also hybridized to RNA molecules with sizes of 3.2 and 5.0 kb. Genomic DNA analysis revealed that the apple PGIP probably belongs to a small family of genes. PGIP transcript levels varied in fruit collected at different maturities, suggesting the gene is developmentally regulated. Very high PGIP transcript levels were detected in decayed areas and the tissue adjacent to the inoculation sites of *Penicillium expansum* and *Botrytis cinerea*. However, no increase in the amount of PGIP transcript in tissue distant from the decayed region was observed. Wounding on fruit also induced PGIP gene expression but to a much lessser extent when compared with decayed areas. After storage at 0 °C for 1 month, the abundance of PGIP transcript in ripe fruit was substantially increased. The PGIP gene in immature and ripe fruit was rapidly up-regulated by fungal infections, while in stored fruit the induction was very limited and concurred with an increase of fruit susceptibility to fungal colonization. Since PGIP gene expression is regulated by fruit development and responds to wounding, fungal infection and cold storage, these observations suggest that apple PGIP may have multiple roles during fruit development and stress response.

Introduction

The plant cell wall, composed of complex polysaccharides, phenolics and structural proteins, maintains cell and tissue integrity and protects the cell from the hostile outside environment. Pectin is the major matrix in the middle lamella and primary cell wall, with a backbone composed of alternating homogalacturonans and rhamnogalacturonans. In order to penetrate and metabolize the cell wall, most microorganisms produce a broad spectrum of cell wall-degrading enzymes [8, 36]. Of the multiple enzymes involved in the degradation of different cell wall components, those breaking down pectic polymers have undergone the most intensive investigation.

Polygalacturonase (PG; EC 3.2.1.15), hydrolyzing the $\alpha 1 \rightarrow 4$ linkages between galacturonic acids in homogalacturonans, is the first enzyme secreted by plant fungal pathogens when cultured on isolated cell walls [18]. Pretreatment of the cell wall with PG appears to facilitate the ability of other cell wall-degrading

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number U77041.

enzymes to attack their substrates [20]. PG can be isolated from infection sites and can spread into host tissue in advance of the invading fungal mycelium [10, 40]. Degradation of pectic components in the plant cell wall and middle lamella by purified PG results in the separation of cells and maceration of host tissue, which is the typical symptom caused by soft-rot pathogens. Based on these observations, a role for PG in pathogenicity has been proposed for soft-rot pathogens [3, 8]. Plant cell walls also contain proteins which specifically and effectively inhibit PGs of fungal origin. These polygalacturonase-inhibiting proteins (PGIP) have been found in the cell wall of all dicotyledonous plants that have been examined [8, 17], and genes which encode these proteins have been cloned from several species [16, 32, 33, 34]. When the pear PGIP gene was constitutively expressed in tomato using the CaMV 35S promoter, fruit from greenhouse-grown primary transgenic plants and their first-generation progeny were more resistant to *Botrytis cinerea* infection than control fruit [25]. However, overexpression of the bean pgip-1 gene in tomato plants did not enhance plant disease resistance against fungal infection [11].

B. cinerea and *Penicillium expansum* are typical soft-rotting pathogens and cause significant losses of apple fruit in storage. The implication that PGIP may be involved as a plant defense mechanism indicates its potential as an alternative method to control postharvest diseases. We have purified and characterized an apple PGIP from mature Golden Delicious apple fruit (*Malus domestica* Borkh.) [39]. In this paper, we report the cloning of an apple PGIP cDNA, and its gene activation by fungal infections and other environmental stresses.

Materials and methods

Fungal and plant materials

B. cinerea and *P. expansum* were isolated from naturally infected apple fruit and maintained on potato dextrose agar. Golden Delicious apple fruit were collected from a commercial orchard in Pennsylvania at two-week intervals during the 1997 growing season. Ripe fruit were harvested on 24 September and stored at 0 °C. Fruit at different maturities and ripe fruit stored for 1 month were used for the study. Cortical tissue, including the peel, was used to determine the amount of PGIP transcripts. Fruit were wounded and inoculated with conidia of either *B. cinerea* or *P. expansum* at a concentration of 1×10^5 spores/ml following the method described previously [9]. Wounded fruit dipped in water and healthy fruit were used as controls. Fruit were kept in a 20 °C incubation room. Disease development was evaluated and apple tissue from different parts of the decayed area was collected to determine the PGIP gene expression level.

Nucleic acids extraction

DNA was isolated from young apple leaves following the procedures described by Doyle and Doyle [15], and the concentration was determined using a fluorometer (Hoefer, San Francisco, CA). RNA was extracted from apple fruit using the protocol of Verwoerd *et al.* [35] with modifications. Six ml of extraction buffer (0.1 M LiCl, 0.1 M Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS, 1% PVP-40 and 1% 2-mercaptoethanol) were mixed with an equal volume of phenol saturated with Tris-HCl pH 8.0 (Sigma, St. Louis, MO). The solution was heated in an 80 °C water bath, then mixed with 4 g of powdered tissue. The mixture was vortexed for 0.5 min, 6 ml of chloroform was added, and vortexed again. The mixture was centrifuged at $12\,100 \times g$ for 10 min at 4 °C and the upper aqueous phase was transferred to a new tube and mixed with an equal volume of 4 M LiCl. RNA sample was placed at −20 °C overnight to freeze the mixture. The sample was thawed and centrifuged as above for 30 min. The RNA pellet was dissolved in 200 μl of DEPC-treated water, and mixed with 20 μl of 3 M sodium acetate pH 5.2. The sample was extensively centrifuged at $15\,800 \times g$ for 1 h to remove the contaminating polysaccharides. RNA was recovered from the supernatant by ethanol precipitation.

PCR amplification and cloning

Three degenerate oligonucleotides, one derived from the determined N-terminal sequence CNPDDKKV [39], and the other two based on the pear PGIP protein sequences CLCGAP and CGQIPVG, were synthesized. The three oligonucleotides are named: PGIP-N1, TGYAAYCCNGAYGAYAARAARGT; PGIP-C1, GGNGCNCCRCANAGRCA; and PGIP-C2, CCNACNGGDATYTGNCCRCA. PCR amplifications of 50 ng of apple genomic DNA were conducted in a Perkin-Elmer Cetus Thermal Cycler. Reaction conditions were as follows: 94 °C (4 min), 52 °C (0.5 min), and 72 °C (1.5 min) for one cycle, then 94 °C (1 min),

52 °C (0.5 min), and 72 °C (1.5 min) for 39 cycles. The final cycle ended with 10 min at 72 °C. The PCR products were excised from low-melting agarose gel and purified according to published protocols [28]. Cloning and sequence analysis of PCR products were performed as described previously [40]. Clone pPIN2 containing the PCR fragment generated by primers PGIP-N1 and C1 was used as a probe to screen a cDNA library derived from poly(A)$^+$ RNA of ripe apple fruit as described by Ross *et al.* [27]. Inserts of putative positive clones were analyzed. A clone which contains the entire coding region of apple PGIP was isolated for characterization. DNA sequence analysis was performed using an ABI automated DNA sequencer.

Gel blot analyses

RNA electrophoresis was performed following the standard protocol [29]. DNA samples were separated on 1% agarose gels in 0.5× TBE [28]. Gels were blotted onto Hybond N$^+$ membranes (Amersham Corp., Arlington Heights, IL) in 10× SSC with a vacuum blotter according to the procedures described by the manufacturer (BioRad, Hercules, CA). Nucleic acids were UV-crosslinked to membranes using a BioRad GS Gene Linker. Antisense RNA probes were generated with a DIG-RNA labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). The membranes were prehybridized for 2 h in 5× SSC, 2% blocking reagent (Boehringer Mannheim), 0.1% sodium lauroylsarcosine, 0.2% SDS, and 50% formamide. The hybridization solution was prepared by adding the RNA probe to a final concentration of 50 ng/ml in fresh prehybridization solution. Prehybridizations and hybridizations were performed at 55 °C for DNA analysis, and 68 °C for RNA. Membranes were washed at room temperature for 15 min in 1× SSC and 0.1% SDS twice, then at 65 °C (DNA blot) or 68 °C (RNA blot) for 15 min in 0.1× SSC and 0.1% SDS twice. Hybridization signals were detected using a Genius 7 Luminescent Detection Kit according to the procedures recommended by the manufacturer (Boehringer Mannheim). After the addition of chemiluminescent substrate CSPD (Boehringer Mannheim), membranes were exposed to Kodak X-Omat AR film at room temperature to detect hybridization signals. The signal intensity for the PGIP transcript in each sample was measured using Stratagene's Eagle Eye II still video system (La Jolla, CA). Differences in RNA loading on the blot were corrected using a 17S rRNA probe from *Neurospora crassa*.

Results

Cloning and analysis of apple PGIP cDNA

The determined N-terminal amino acid sequence of apple PGIP showed 96% identity with pear PGIP, with only one difference across 25 amino acids [39]. Based on this information, an 8 amino acid apple PGIP N-terminal sequence, and two stretches of amino acid sequences at the C-terminus of pear PGIP sharing a high degree of homology with other PGIPs, were used to design degenerate oligonucleotides PGIP-N1, PGIP-C1, and PGIP-C2. When PGIP-N1 and C1 were used as primers to amplify apple genomic DNA by PCR, a fragment of 896 bp was produced. To confirm that the fragment was derived from the PGIP gene, it was reamplified using PGIP-N1 and C2 as primers. As expected, this resulted in the production of a fragment which was slightly shorter (833 bp). Both fragments were cloned, and designated pPIN1 (833 bp) and pPIN2 (896 bp). Sequence analyses confirmed that both fragments showed high homologies with other cloned PGIPs [16, 32, 33, 34], and a perfect match to each other except in the primer regions. pPIN2 was used as a probe to screen an apple cDNA library. Three positive clones were identified, and the one with the largest insert was named pPGIP1 and sequenced. The nucleotide sequence of the pPGIP1 clone was 1162 bp long with an open reading frame encoding a predicted polypeptide of 330 amino acids (Figure 1). The first 24 amino acids were identified as the signal peptide, based on the known N-terminal sequence. The N-terminal amino acid sequence of the deduced mature protein matched perfectly with the determined amino acid sequence, suggesting it is derived from the authentic PGIP gene transcript. The predicted mature protein has an isoelectric point of 7.0, and a calculated molecular mass of 34 kDa. The full-length apple PGIP mRNA was determined to be 1.3 kb by northern blot analysis (data not shown).

Apple PGIP and other related sequences

A comparison of apple PGIP with other available PGIP sequences in databases using the BESTFIT program [12] revealed that this clone showed 97, 71, 71, 66, 63, and 61% identities at the DNA sequence level, and 98, 83, 82, 81, 67 and 63% identities at

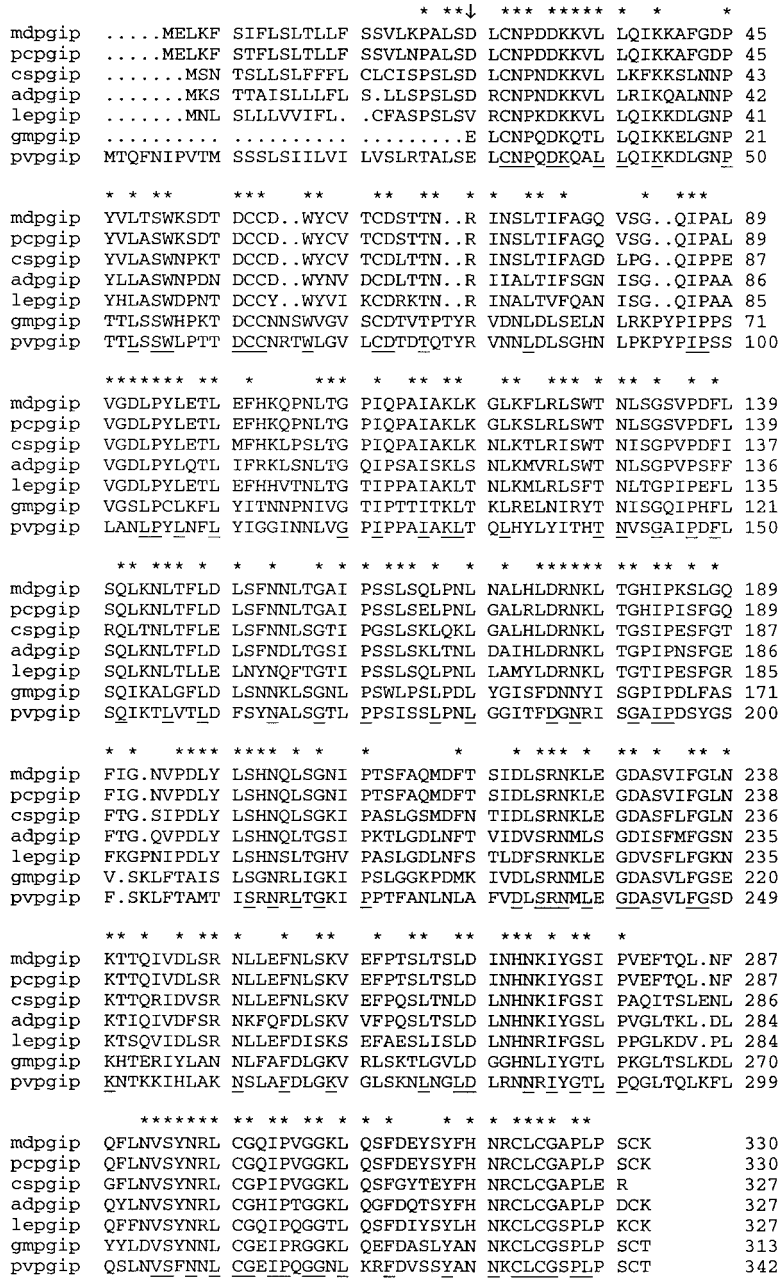

Figure 1. Alignment of all characterized PGIP deduced amino acid sequences. Asterisks indicate identical amino acids among PGIPs isolated from fruit. Underlined amino acids are the invariant residues among all PGIPs. The arrow indicates the N-terminal amino acid of the mature PGIP polypeptides. Numbers on the right are the coordinates of each PGIP. Each PGIP is named using its genus and species initials: apple, *Malus domestica*; pear, *Pyrus communis* [33]; orange, *Citrus sinensis* (accession number Y08618); kiwifruit, *Actinidia deliciosa* [30]; tomato, *Lycopersicon esculentum* [32]; soybean, *Glycine max* [16]; bean, *Phaseolus vulgaris* [34].

Figure 2. Domain structure of apple PGIP. Invariant amino acids composed of leucine-rich repeat (LRR) sequence motifs are shown in bold.

the amino acid sequence with those from pear [33], orange (accession number Y08618), kiwifruit [30], tomato [32], bean [34], and soybean [16], respectively. The deduced apple PGIP polypeptide sequence is more homologous to those derived from pear, orange, or kiwifruit than those from bean and soybean. While processed PGIPs from fruit shared 53% identical amino acids, the percentage of overall identity was reduced to only 32% when PGIPs from bean and soybean were included in the analysis (Figure 1). The locations of 8 cysteine residues were invariant among all PGIP polypeptides, with one exception in orange, where the last cysteine at the end of coding region was absent. While fruit PGIPs have 6 to 7 putative N-glycosylation sites, PGIPs from bean and soybean have only 3 to 4 potential sites in their predicted mature polypeptides. Only one of these sites, which corresponds to amino acid 130 on the apple PGIP molecule, is conserved among all inhibitors. However, fruit PGIPs share 4 such common sites, which are located at positions 130, 144, 238 and 291 on the apple PGIP. The signal peptide cleavage site is also conserved, with cleavage after the amino acid serine (S). The N-terminal amino acid of mature PGIPs from fruit is aspartic acid (D), except in tomato PGIP, which is valine (V), and glutamic acid (E) for both bean hypocotyl and soybean seedling PGIPs (Figure 1). Interestingly, the N-terminal residue of bean pod PGIP is also aspartic acid, as suggested by its fruit nature [26]. The majority of other invariant amino acids among PGIPs are those involved in the formation of leucine-rich repeat (LRR) sequence motifs as described below.

Additional protein sequence comparisons were done using the BLAST program [2] on the network (National Center for Biotechnology Information, Behesda, MD). In addition to showing high homologies with other PGIPs, apple PGIP also showed good matches with diverse groups of proteins containing leucine-rich repeats (LRRs). Among them, the greatest homologies were detected with factors responsible for plant disease resistance and signal transduction, such as the products of tomato *Cf-2*, *Cf-9* genes [14, 19], *Arabidopsis RPS2* genes [4, 24], tobacco *N* gene [38], flax *L6* gene [23], and receptor-like protein kinase genes from rice and *Arabidopsis thaliana* [7, 31, 37]. The LRR core region of apple PGIP is composed of ten imperfect repeats with an average of 24 amino acids (Figure 2). The LRR consensus sequence for apple PGIP is -L- -L-LS-N-L-G-IP- -A- -L-. In this consensus, A represents aliphatic amino acids (A, V, L, I, F, Y or M), which are present at the position in more than 80% of the repeats, a gap (−) represents any amino acid, and an invariant amino acid in the consensus represents its presence at that position in more than half of the repeats. This consensus sequence is shared by other PGIPs and also the gene products of disease resistance and signal transduction factors described above. However, the conserved amino acid leucine (L) can frequently be substituted by the equivalent hydrophobic isoleucine (I).

Genomic organization of PGIP gene

Apple genomic DNA was used to perform Southern analysis to determine the presence of other PGIP homologous sequences. DNA was digested with *Eco*RI, *Hin*dIII, *Xba*I, *Eco*RV, *Sac*I and *Sca*I, and hybridized with the digoxigenin-labeled RNA probe generated from *Eco*RI-linearized pPIN2. Multiple bands were observed for each enzyme tested, including *Eco*RI,

*Sac*I and *Sca*I, which have no recognition site in the pPIN2 probe. This suggests that the apple genome has at least two or a small family of PGIP homologous genes (Figure 3).

PGIP gene expression during fruit development and in response to stresses

Three independent RNA extractions from apple fruit collected at different developmental stages and ripe fruit stored for 1 month at 0 °C were used to conduct northern analyses. Gel blots were first hybridized with the probe pPIN2 to determine the amount of PGIP gene transcripts. After stripping the probe, the same blots were subjected to hybridization with an RNA probe generated from the 17S rRNA gene of *N. crassa*. The PGIP probe mainly hybridized with RNA molecules of 1.3 kb, which is the expected size of the full-length apple PGIP gene transcript. In addition, the probe also hybridized to transcripts of 3.2 and 5.0 kb, which were particularly evident in the RNA sample extracted from stored fruit (Figure 4A). The hybridization signals from apple PGIP transcripts were quantified, and standardized with corresponding rRNA levels. There was a 3-fold variation in PGIP transcript levels in fruit at different developmental stages (Figure 4B). The PGIP transcript level was the lowest in immature fruit collected two weeks before harvest. After storage at 0 °C for 1 month, accumulation of the PGIP transcript was increased by more than 2-fold, when compared to fresh ripe fruit (Figure 4B). During fruit development and storage, the apples became more susceptible to fungal infection and tissue maceration as indicated by the increased lesion size on the fruit inoculated with *P. expansum* or *B. cinerea* (Table 1). There was no correlation between the lesion size and the amount of PGIP transcripts detected in healthy fruit.

The induction of apple PGIP gene expression by fungal infections and tissue wounding was also analyzed. Fruit were wounded on two sides to a depth of 2 mm by being pressed down on a nail head of 2 mm diameter. Fruit were then immersed in water for wounding treatment or in fungal spore suspensions for inoculation. Healthy fruit were used as a control. The data presented was generated from fresh ripe fruit collected on 24 September. After incubation at 20 °C for 6 days, apple tissue from decayed and adjacent areas, as well as tissue distant from the decayed region on the inoculated fruit was collected. Tissue from the wounded region as well as from healthy fruit was also collected. Total RNA isolated from these samples was used to conduct northern blot analyses using RNA probes derived from the apple PGIP cDNA and fungal 17S rRNA (Figure 5A). While wounding induced a 7-fold increase in the level of PGIP mRNA detected, even higher levels of PGIP mRNA were detected in the decayed and adjacent areas on inoculated fruit (Figure 5B). In *B. cinerea*-rotted fruit, about 14-fold increases in the PGIP transcript levels were detected in both the decayed and adjacent regions. In the same regions on *P. expansum*-inoculated fruit, there were about 11-fold increases in the amount of PGIP transcripts (Figure 5B). However, in the regions distant from the decayed area, there were no substantial changes in the amount of transcripts detected. In

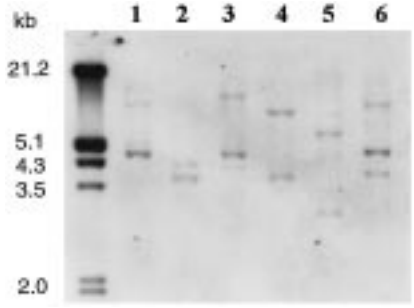

Figure 3. Southern blot analysis of PGIP in apple genomic DNA. DNA was digested with *Eco*RI (lane 1), *Hin*dIII (lane 2), *Xba*I (lane 3), *Eco*RV (lane 4), *Sac*I (lane 5), and *Sca*I (lane 6), and hybridized with digoxigenin-labeled pPIN2. Lane on the far left side contains *Eco*RI- and *Hin*dIII-digested lambda DNA labeled with digoxigenin. Sizes of the standard DNA fragments are indicated in kb.

Table 1. Fruit weight and size of the decayed area caused by *Botrytis cinerea* and *Penicillium expansum* on apple fruit collected at different maturities[1].

Date	Fruit Weight (g)	*B. cinerea*[2] decay (cm)	*P. expansum*[2] decay (cm)
8/13	99.96 ± 8.9	0.98 ± 0.39^{a}	1.69 ± 0.34^{a}
8/27	117.78 ± 11.62	1.42 ± 0.80^{b}	2.03 ± 0.36^{b}
9/11	140.79 ± 19.88	1.42 ± 0.72^{b}	1.99 ± 0.37^{b}
9/24	145.36 ± 18.64	2.67 ± 1.10^{c}	2.51 ± 0.51^{c}
10/24[3]	145.21 ± 15.76	3.73 ± 0.91^{d}	2.81 ± 0.53^{d}

[1]Data are expressed as mean ± standard deviation. 20 fruit were used to determine fresh weight and test fungal pathogenicity. Decay was expressed as the average diameter of 40 lesions. No infection was counted as 0.
[2]Different superscripts indicate that the lesion sizes were significantly different at $P < 0.01$.
[3]Ripe fruit after 1 month of storage at 0 °C.

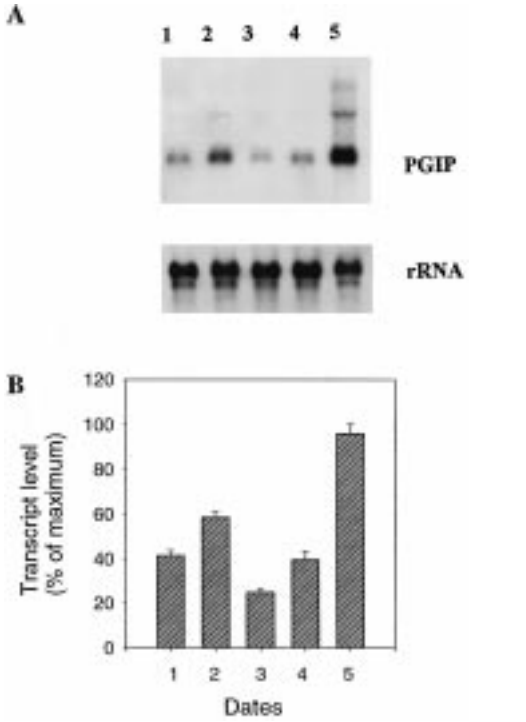

Figure 4. PGIP gene expression level in healthy apple fruit at different maturities. Total RNA (2 μg) extracted from fruit collected on 13 August (lane 1), 27 August (lane 2), 11 September (lane 3), 24 September 1997 (lane 4), and ripe fruit stored for 1 month at 0 °C (lane 5) was used to perform gel blot hybridizations. The membranes were first hybridized to pPIN 2, then hybridized to a *Neurospora crassa* 17S rRNA probe after stripping of the old probe. Autoradiographs derived from the hybridizations are shown in A. The quantification of the apple PGIP transcript level is shown in B, which is corrected with the rRNA level. The error bar represents the standard deviation in signal intensities derived from three independent RNA preparations.

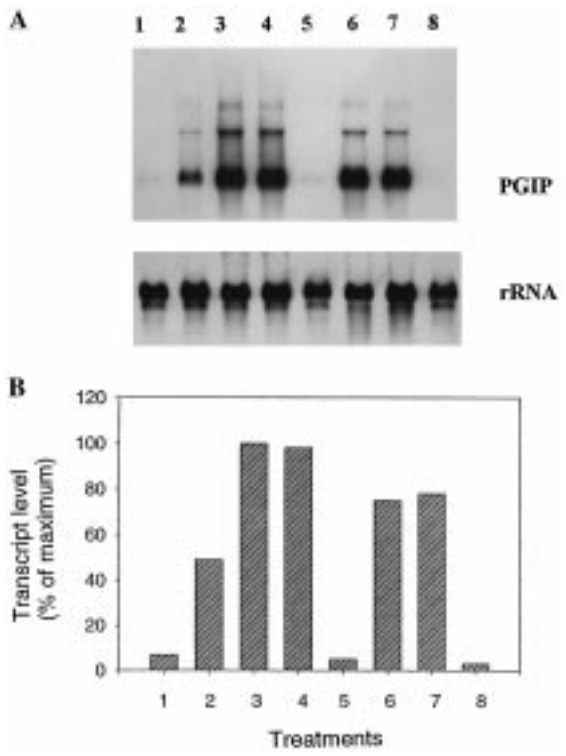

Figure 5. PGIP gene activation in fresh ripe apple fruit in response to tissue wounding and infections by *Penicillium expansum* and *Botrytis cinerea*. RNA was isolated from healthy fruit (lane 1), wounded area (lane 2), *B. cinerea*-decayed (lane 3), adjacent to (lane 4) and distant from decayed areas (lane 5), *P. expansum*-decayed (lane 6), adjacent to (lane 7) and distant from decayed areas (lane 8) 6 days after inoculation. Total RNA (2 μg) was used for gel blot hybridizations. The membrane was first hybridized with pPIN 2, then hybridized with a *Neurospora crassa* 17S rRNA probe following removal of the old probe. Autoradiographs derived from the hybridizations are shown in A. The quantification of the apple PGIP transcript level is shown in B, which is corrected with the rRNA level.

summary, these results indicated that the apple PGIP gene was strongly activated locally in the decayed and surrounding areas but not systemically in tissue distant from inoculated sites in response to mechanical damage and pathogen challenge. RNA blot hybridization revealed that the 3.2 bp transcript abundance was also induced in a manner parallel to PGIP gene activation. Similar gene induction patterns were observed on immature fruit in response to these biotic and abiotic stresses (data not shown). However, side-by-side comparison using RNA extracted from stored and fresh ripe fruit showed that PGIP gene induction by wounding and fungal infections in stored ripe fruit was very limited (Figure 6A). In stored ripe fruit, there was only a marginal increase in the PGIP transcript level in the wounded region. In the decayed regions caused by *P. expansum* and *B. cinerea*, about 2-fold increases in the PGIP transcript levels were detected respectively (Figure 6B). In striking contrast, much higher levels of PGIP transcript accumulated in fresh ripe fruit challenged by wounding and fungal pathogens. Although high basal levels of PGIP transcript in stored healthy fruit reduced the potential for further up-regulation in treated stored fruit, there was 2- to 3-fold more PGIP mRNA present in the respectively challenged fresh ripe fruit (Figure 6B). The lower levels of induction by wounding and fungal infection in stored fruit coincided with a significant increase of fruit susceptibility to fungal colonization and maceration of host tissue (Table 1).

Temporal PGIP gene expression in healthy, wounded and inoculated tissues of fresh ripe fruit was

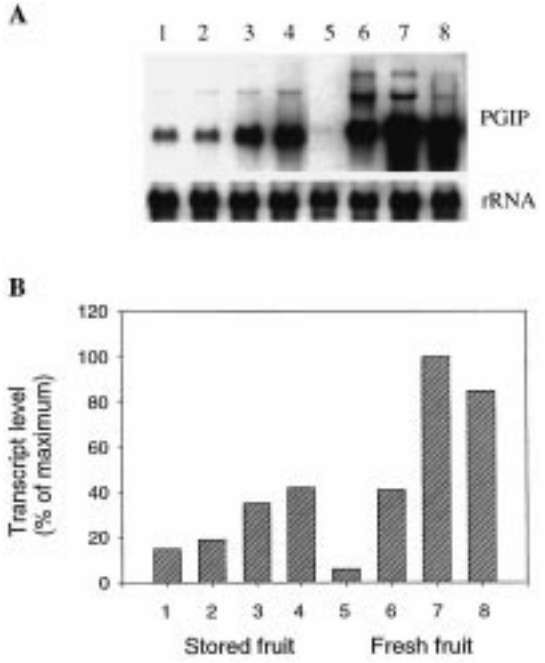

Figure 6. PGIP gene induction in stored and fresh ripe fruit. RNA samples in lanes 1 to 4 are from stored fruit, those in lanes 5 to 8 are from fresh ripe fruit. RNA was extracted from healthy (lanes 1, 5), wounded (lanes 2, 6), *Botrytis cinerea* (lanes 3, 7), and *Penicillium expansum* (lanes 4, 8) decayed apple tissue 7 days after inoculation. Total RNA (2 μg) was electrophoresed and blotted onto the membrane. The blot was hybridized to a PGIP probe pPIN2 to determine the abundance of PGIP transcript in samples. The same blot was hybridized with a fungal 17S rRNA probe to ensure equal loading of RNA samples. Autoradiographs derived from gel blot hybridization are shown in A. The graph representing apple PGIP transcript level is shown in B.

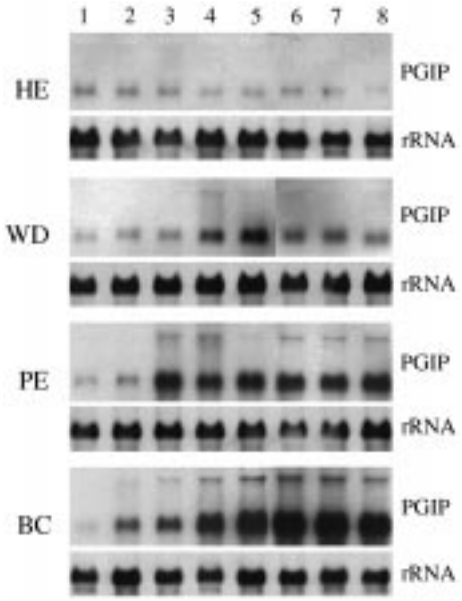

Figure 7. Temporal expression pattern of the PGIP gene in response to wounding and fungal infections in fresh ripe fruit. RNA was isolated from healthy (HE), wounded (WD), *Penicillium expansum* (PE) and *Botrytis cinerea* (BC) inoculated fruit at 0 (lane 1), 1 (lane 2), 2 (lane 3), 3 (lane 4), 4 (lane 5), 5 (lane 6), 6 (lane 7), and 7 days (lane 8) after inoculation. Total RNA (2 μg) was electrophoresed and blotted onto the membrane. The blot was hybridized to a PGIP probe pPIN2 to determine the abundance of PGIP transcript in samples. The same blot was hybridized with a fungal 17S rRNA probe to ensure equal loading of RNA samples.

analyzed (Figure 7). Tissue was collected from day 0 to day 7 at 24 h intervals, and RNA was isolated from each tissue sample. RNA blot hybridization with pPIN2 showed that there were no significant changes in PGIP transcript levels in the healthy fruit, although a slight decrease in the hybridization signal was noticed in fruit kept longer at room temperature. In the wounded area, the accumulation of PGIP mRNA increased steadily, reaching the highest level by day 4 and then leveled off gradually. In the *P. expansum*- and *B. cinerea*-inoculated regions, the increases of PGIP gene expression were evident 1 day after inoculation. In the case of the *P. expansum* challenge, PGIP transcript levels reached the highest level by day 2, and a similar level of PGIP transcript was maintained in the infected tissue as disease developed. In *B. cinerea*-inoculated fruit, more PGIP mRNA was detected than in *P. expansum*-challenged fruit. PGIP transcript levels gradually increased in the *B. cinerea*-inoculated fruit, reaching its highest level by day 5 and then leveled off.

Discussion

A cDNA encoding an apple polygalacturonase inhibitor was cloned. Its identity as apple PGIP was confirmed by the perfect match between the determined N-terminal amino acid sequence and the deduced polypeptide sequence, as well as the high homologies with other characterized PGIPs. The predicted molecular mass is the same as the estimated molecular mass for the native protein following chemical removal of associated oligosaccharides [39]. The predicted isoelectric point derived from the clone was 7.0, while native protein was mainly electrofocused in the pH range 3.0 to 5.9 [39]. This discrepancy could be due to the differential glycosylation of seven potential N-glycoslation sites on the same polypeptide molecule.

PGIP belongs to a group of proteins containing LRRs, with diverse function and cellular location [22]. However, these sequence motifs have been found to be mainly involved in protein-protein or protein-ligand interactions. The three-dimensional structure of one of these proteins, porcine ribonuclease inhibitor (PRI), has been determined [21]. In the PRI molecule, LRRs correspond to β-α structural units, consisting of a short β-strand and an α-helix approximately parallel to each other, resulting in a nonglobular, horseshoe-shaped molecule [21]. Binding of PRI to ribonuclease was extensive and involved multiple amino acid residues, which were probably located on the surface formed by the β-sheat and $\beta\alpha$ loops. Like PRI, the PGIP molecule is mainly composed of LRRs. Therefore, it could adapt to a similar three-dimensional structure, and inhibit fungal PG by extensive binding to its surface amino acid residues. This can give a plausible explanation of the observed differential inhibition of PGIP to fungal PGs. The binding strength between two molecules may determine the degree to which PG can be inhibited by PGIP.

The PGIP gene was constitutively expressed in apple fruit during development. Variations in the PGIP transcript levels in fruit at different developmental stages were reproducibly observed. The gene expression pattern was attributed to the developmental regulation rather than due to fruit handling, since similar results were obtained when experiments were carried out in the United States and New Zealand. It is tempting to speculate that plant hormones such as ethylene would likely be involved in the control of PGIP gene expression. However, we do not know the nature of the regulatory factor at present. A correlation between the PGIP transcript levels in healthy fruit and the lesion sizes on inoculated fruit with *P. expansum* and *B. cinerea* was not established, suggesting that the constitutive expression of apple PGIP in healthy fruit may not be related to disease resistance, but may have a role in the normal growth and development of fruit.

It has been demonstrated that PGIP gene products accumulated in bean in response to wounding, elicitors and fungal infection [5, 13]. In pear and tomato fruits, it has been reported that PGIP genes were constitutively expressed and were not induced by wounding or pathogen challenge [25]. However, we found that mechanical damage and fungal infection of apple tissue can efficiently activate the local expression of the apple PGIP gene in immature and fresh ripe fruit, though this inducibility was substantially reduced in stored fruit. These results suggest that apple PGIP may be actively involved in the defense mechanisms of fruit against pathogen infection. Pathogen-mediated induction of the PGIP gene was more extensive and reached a higher level in all tests. Although these observations could be attributed to more extensive tissue damage caused by fungal pathogens in later stages of disease development, the accumulation of PGIP mRNA in *P. expansum*- and *B. cinerea*-inoculated fruit was much higher than in wounded tissue 24 h after inoculation. In this early stage, fungal spores had just began to germinate and the damage to host tissue was very limited. This suggests that fungal pathogens also released some signals(s) that activated the PGIP gene. Fungal PG could be one of these signals. In plant-fungal interactions, oligosaccharides derived from PG degradation of cell wall pectin have been found to be potent elicitors of the plant defense response [1]. It has been proposed that bean PGIP can lead to the accumulation of elicitor-active oligogalacturonides by slowing down the process of oligosaccharide depolymerization by PG [6]. Apple PGIP may also have a similar function in the apple fruit-fungal molecular interaction.

Southern analysis under high-stringency conditions revealed the presence of more than one homologous PGIP gene in apple. In RNA gel blot analysis, in addition to the detection of the 1.3 kb PGIP mRNA, the probe also hybridized with gene transcripts of 5.0 and 3.2 kb. These transcripts were present in healthy fruit, and their levels increased in response to fungal infections, wounding and cold storage. These data suggest that they could be the gene transcripts related to PGIP, though we can not exclude the possibility of hybridization artifacts.

While tomato fruit from transgenic plants overexpressing the pear PGIP gene did show higher resistance to *B. cinerea* infection [25], disease resistance to several pathogenic fungi in tomato plants with bean *pgip-1* constitutively expressed was not improved [11]. In our study, we found that the expression of the apple PGIP gene was developmentally regulated, and its transcriptional activation in fruit tissue can be induced by several environmental stresses such as cold storage, mechanical wounding and fungal infection. These results suggest that plant PGIP may have multiple functions both in normal plant development and in response to biotic and abiotic stresses. The presence of multiple PGIP genes in plants such as bean, tomato and pear has been reported [11, 32, 33]. Overexpression of one member of the bean PGIP family was not sufficient to enhance disease resistance in transgenic

tomato plants [11]. Therefore, in order to gain insight concerning the role of PGIP in plants, characterization of different PGIP genes in a single plant will be a prerequisite. Transgenic work where PGIP activity is completely removed from a plant by expressing an antisense gene structure or by cosuppression with the introduction of multiple copies of the PGIP gene may provide us with more direct evidence regarding its role in fruit development and stress response.

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References

1. Albersheim P, Darvill A, Augur C, Cheong J, Eberhard S, Hahn MG, Marfa V, Mohnen D, O'Neill MA, Spiro MD, York WS: Oligosaccharins: oligosaccharide regulatory molecules. Acc Chem Res 25: 77–83 (1992).
2. Altschul SF, Gish W, Millar W, Myers EW, Lipman DJ: Basic local alignment search tool. J Mol Biol 215: 403–410 (1990).
3. Bateman DF, Millar RL: Pectic enzymes in tissue degradation. Annu Rev Phytopath 4: 119–146 (1966).
4. Bent AF, Kunkel BN, Dahlbeck D, Brown KL, Schmidt R, Giraudat J, Leung J, Staskawicz BJ: *RPS*2 of *Arabidopsis thaliana*: a leucine-rich repeat class of plant disease resistance genes. Science 265: 1856–1860 (1994).
5. Bergmann CW, Ito Y, Singer D, Albersheim P, Darvill AG, Benhamou N, Nuss L, Salvi G, Cervone F, De Lorenzo G: Polygalacturonase-inhibiting protein accumulates in *Phaseolus vulgaris* L. in response to wounding, elicitors and fungal infection. Plant J 5: 625–634 (1994).
6. Cervone F, Hahn MG, De Lorenzo G, Darvill A, Albersheim P: Host-pathogen interactions. XXXIII. A plant protein concerts a fungal pathogenicity factor into an elicitor of plant defense responses. Plant Physiol 90: 542–548 (1989).
7. Chang C, Schaller GE, Patterson SE, Kwok SF, Meyerowitz EM, Bleecker AB: The TMK1 gene from *Arabidopsis* codes for a protein with structural and biochemical characteristics of a receptor protein kinase. Plant Cell 4: 1263–1271 (1992).
8. Collmer A, Keen NT: The role of pectic enzymes in plant pathogenesis. Annu Rev Phytopath 24: 383–409 (1986).
9. Conway WS: Effect of postharvest calcium treatment on decay of Delicious apples. Plant Dis 66: 402–403 (1982).
10. Cooper RM: Pathogen-induced changes in host ultrastructure. In: Staples RC, Toenniesen CH (eds) Plant Disease Control: Resistance and Susceptibility, pp. 105–142. John Wiley, New York (1981).
11. Desiderio A, Aracri B, Leckie F, Mattei B, Salvi G, Tigelaar H, van Roekel JSC, Baulcombe DC, Melchers LS, De Lorenzo G, Cervone F: Polygalacturonase-inhibiting proteins (PGIPs) with different specificities are expressed in *Phaseolus vulgaris*. Mol Plant-Microbe Interact 10: 852–860 (1997).
12. Devereux J, Haeberli P, Smithies O: A comprehensive set of sequence analysis programs for the VAX. Nucl Acids Res 12: 387–395 (1984).
13. Devoto A, Clark AJ, Nuss L, Cervone F, De Lorenzo G: Developmental and pathogen-induced accumulations of transcripts of polygalacturonase-inhibiting protein in *Phaseolus vulgaris* L. Planta 202: 284–292 (1997).
14. Dixon MS, Jones DA, Keddie JS, Thomas CM, Harrison K, Jones JDG: The tomato *Cf-2* disease resistance locus comprises two functional genes encoding leucine-rich repeat proteins. Cell 84: 451–459 (1996).
15. Doyle JJ, Doyle JL: Isolation of plant DNA from fresh tissue. Focus 12: 13–15 (1990).
16. Favaron F, D'Ovidio R, Porceddu E, Alghisi P: Purification and molecular characterization of a soybean polygalacturonase-inhibiting protein. Planta 195: 80–87 (1994).
17. Hahn MG, Bucheli P, Cervone F, Doares SH, O'Neill RA, Darvill AG, Albersheim P: The roles of cell wall constituents in plant-pathogen interactions. In: Nester E, Kosuge T (eds) Plant-Microbe Interactions, vol 3, pp. 131–181. Macmillan, New York (1989).
18. Jones TM, Anderson AJ, Albersheim P: Host-pathogen interaction. IV. Studies on the polysaccharide-degrading enzymes secreted by *Fusarium oxysporum* f. sp. *lycopersici*. Physiol Plant Path 2: 153–166 (1972).
19. Jones DA, Thomas CM, Hammond-Kosack KE, Balint-Kurti PJ, Jones JDG: Isolation of the tomato *Cf-9* gene for resistance to *Cladosporium fulvum* by transposon tagging. Science 266: 789–793 (1994).
20. Karr AL, Albersheim P: Polysaccharide-degrading enzymes are unable to attack cells without prior action by a 'wall-modifying' enzyme. Plant Physiol 46: 69–80 (1970).
21. Kobe B, Deisenhofer J: Crystal structure of porcine ribonuclease inhibitor, a protein with leucine-rich repeats. Nature 366: 751–756 (1993).
22. Kobe B, Deisenhofer J: The leucine-rich repeat: a versatile binding motif. Trends Biochem Sci 19: 415–421 (1994).
23. Lawrence GJ, Finnegan EJ, Ayliffe MA, Ellis JG: The *L6* gene for flax rust resistance is related to the Arabidopsis bacterial resistance gene *RPS*2 and the tobacco viral resistance gene *N*. Plant Cell 7: 1195–1206 (1995).
24. Mindrinos M, Katagiri F, Yu G, Ausubel FM: The *A. thaliana* disease resistance gene *RPS*2 encodes a protein containing a nucleotide-binding site and leucine-rich repeats. Cell 78: 1089–1099 (1994).
25. Powell ALT, Stotz HU, Labavitch JM, Bennett AB: Glycoprotein inhibitors of fungal polygalacturonases. In: Daniel *et al.* (eds) Advances in Molecular Genetics of Plant-Microbe Interactions 3, pp. 399–402. Kluwer Academic Publishers, Dordrecht, Netherlands (1994).
26. Pressey R: Polygalacturonase inhibitors in bean pods. Phytochemistry 42: 1267–1270 (1996).
27. Ross GS, Knighton ML, Lay-Yee M: An ethylene-related cDNA from ripening apples. Plant Mol Biol 19: 231–238 (1992).
28. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989).
29. Selden RF: Analysis of RNA by Northern hybridization. In: Ausubel F, Brent R, Kingston R, Moore D, Seidman J, Smith J, Struhl K (eds) Current Protocols in Molecular Biology, pp. 4.9.1–4.9.8. John Wiley, New York (1987).
30. Simpson CG, MacRae E, Gardner RC: Cloning of a polygalacturonase-inhibiting protein from kiwifruit (*Actinidia deliciosa*). Plant Gene Register (PGR95-037). Plant Physiol 108: 1748 (1995).

31. Song W, Wang G, Chen L, Kim H, Pi L, Holsten T, Gardner J, Wang B, Zhai W, Zhu L, Fauquet C, Ronald P: A receptor kinase-like protein encoded by the rice disease resistance gene, *Xa21*. Science 270: 1804–1806 (1995).
32. Stotz HU, Contos JA, Powell ALT, Bennett AB, Labavitch JM: Structure and expression of an inhibitor of fungal polygalacturonases from tomato. Plant Mol Biol 25: 607–617 (1994).
33. Stotz HU, Powell ALT, Damon SE, Greve LC, Bennett AB, Labavitch JM: Molecular characterization of a polygalacturonase inhibitor from *Pyrus communis* L. cv. Barlett. Plant Physiol 102: 133–138 (1993).
34. Toubart P, Desiderio A, Salvi G, Cervone F, Daroda L, De Lorenzo G, Bergmann C, Darvill AG, Albersheim P: Cloning and characterization of the gene encoding the endopolygalacturonase-inhibiting protein (PGIP) of *Phaseolus vulgaris* L. Plant J 2: 367–373 (1992).
35. Verwoerd TC, Dekker BMM, Hoekema A: A small-scale procedure for the rapid isolation of plant RNAs. Nucl Acids Res 17: 2362 (1989).
36. Walton, JD: Deconstructing the cell wall. Plant Physiol 104: 1113–1118 (1994).
37. Walker JC: Receptor-like protein kinase genes of *Arabidopsis thaliana*. Plant J 3: 451–456 (1993).
38. Whitham S, Dinesh-Kumar SP, Choi D, Hehl R, Corr C, Baker B: The product of the tobacco mosaic virus resistance gene *N:* similarity to toll and the interleukin-1 receptor. Cell 78: 1101–1115 (1994).
39. Yao C, Conway WS, Sams CE: Purification and characterization of a polygalacturonase-inhibiting protein from apple fruit. Phytopathology 85: 1373–1377 (1995).
40. Yao C, Conway WS, Sams CE: Purification and characterization of a polygalacturonase produced by *Penicillium expansum* in apple fruit. Phytopathology 86: 1160–1166 (1996).